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# Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor

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## SUMMARY

Cultured normal human fibroblasts were stimulated to produce neutrophil-activating protein/interleukin-8 (IL-8) in response to IL-1α (0·1–1000 U/ml) or tumour necrosis factor (TNF) α (0·1–1000 U/ml). Induction of mRNA for IL-8 in fibroblasts was rapid (within 30 min) and maximal responses were obtained with either 100 U/ml IL-1α or 100 U/ml TNFα. Expression of mRNA for IL-8 was accompanied by the production of high levels of neutrophil chemotactic activity. IL-1α (1000 U/ml), but not TNFα, induced mRNA for IL-8 in cultured normal human keratinocytes. The relevance of production of IL-8 by these cell types was evaluated further by comparing the local inflammatory effects of IL-1α, TNFα and IL-8. Intradermal injection of either recombinant IL-8, IL-1α or TNFα lead to a similar *in vivo* effect, i.e. dose-dependent accumulation of lymphocytes and polymorphonuclear leucocytes at sites of injection. The *in vivo* attraction of neutrophils and lymphocytes to the site of injection by TNF or IL-1 (which is not chemotactic for neutrophils or lymphocytes *in vitro*), may be partly mediated by locally produced IL-8. Thus, IL-8 may be a vital participant in the cascade of interacting cytokines that is induced by tissue injury and immunologically induced inflammation.

## INTRODUCTION

Recruitment of circulating leucocytes such as lymphocytes, monocytes and neutrophils into tissues is dependent on local release of chemoattractant mediators/cytokines during the development of an injury-induced or immunologically mediated inflammatory reaction. A number of mediators, such as IL-1 (Hunninghake *et al.*, 1987), TNFα (Ming, Bersani & Mantovani, 1987), lymphocyte chemotactic factor (Potter & Van Epps, 1987), IL-2 (Potter & Van Epps, 1987), monocyte chemotactic factor (Snyderman *et al.*, 1972; Matsushima *et al.*, 1989; Furutani *et al.*, 1989; Yoshimura *et al.*, 1989), C5a (Snyderman *et al.*, 1969), have been reported to have leucocyte chemotactic properties. Recently, a human neutrophil-activating protein, which is chemotactic for neutrophils, has been purified (Yoshimura *et al.*, 1987a, b; Walz *et al.*, 1987; Van Damme *et al.*, 1988; Schroder, Mrowietz & Christophers, 1987) and cloned (Matsushima *et al.*, 1988). This factor was initially purified from lipopolysaccharide (LPS)-stimulated human monocytes and termed monocyte-derived neutrophil chemotactic factor (MDNCF) (Yoshimura *et al.*, 1987a), but other cell types, such as lymphocytes (Schroder, Mrowietz & Christophers, 1988) and endothelial cells (Schroder & Christophers, 1989) are also able to produce similar factors. In addition, we recently purified a T-lymphocyte chemotactic factor (TCF) from phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMC), established the identity of TCF and MDNCF, and showed the *in vivo* attractive activity of recombinant MDNCF on both lymphocytes and neutrophils (Larsen *et al.*, 1989). In view of its effect on distinct target cells and the production by multiple cell types, we therefore proposed that MDNCF should be renamed interleukin-8 (IL-8) (Larsen *et al.*, 1989; International Meeting of Novel Neutrophil Stimulating Peptides: Source, Structure and Role in Inflammation, London, 1988).

It is well established that proinflammatory mediators such as IL-1 and TNF are produced by a number of cell types. In this report we document that IL-8 is produced by extravascular non-circulating cells such as dermal fibroblasts and keratinocytes in response to IL-1 or TNF. Thus IL-8 appears to be a product of a variety of cell types and to act on distinct target cells.

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## MATERIALS AND METHODS

### *IL-1 and TNF stimulation of fibroblasts in cultures*

Normal human dermal fibroblasts (No. 32-SK; American Type Culture Collection, Rockville, MD) were cultured to subconfluency in Costar 400 ml Tissue culture flasks, using 20 ml DMEM (Advanced Biotechnologies Inc., Columbia, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) and L-glutamine (20 mM) at 37° and 5% $CO_2$. Cells were then stimulated by adding recombinant IL-1$\alpha$ (rIL-1$\alpha$) or recombinant TNF$\alpha$ (rTNF$\alpha$) to the culture media in varying concentrations for different time intervals (0–6 hr). rIL-1$\alpha$ ($2 \times 10^7$ U/mg) were obtained from Dainippon Pharmaceuticals, Osaka, Japan. We used concentrations of IL-1 (0·1–1000 U/ml) and TNF (0·1–1000 U/ml) encompassing physiological relevant levels.

Secondary cultures of normal human keratinocytes were obtained from Clonetics (San Diego, CA) and cultured to subconfluency in Costar 400 ml Tissue culture flask using KGM medium containing bovine pituitary extract (Clonetics), epidermal growth factor (10 ng/ml), hydrocortisone (0·5 $\mu$g/ml), insulin 5 $\mu$g/ml, penicillin, streptomycin, amphotericin-B and calcium (0·15 mM). Based on the optimal conditions for induction of mRNA for IL-8 in fibroblasts (see below), we stimulated keratinocytes for 2 hr with rIL-1$\alpha$ (1000 U/ml) or rTNF$\alpha$ (1000 U/ml).

Following stimulation, culture media were collected and prepared as described below. Total RNA was extracted from adherent cells as previously described (Lew, Oppenheim & Matsushima, 1988).

*Detection of IL-8 mRNA*

Total RNA was blotted onto a Nytran membrane by Northern blotting technique as described elsewhere (Lew *et al.*, 1988). Hybridization, using a $^{32}$P-labelled 0·45 kb EcoR1-EcoR1 fragment IL-8 cDNA probe (Matsushima *et al.*, 1988), was performed and specific hybridization determined by autoradiography (KODAK X-OMAT-AR film, Eastman Kodak, Rochester, NY) for 16 hr.

*Neutrophil chemotactic activity in culture media*

Neutrophil chemotactic activity was detected in conditioned media using an *in vitro* assay as described elsewhere (Falk, Goodwin & Leonard, 1980). Filter-sterilized conditioned media from cultures of fibroblasts stimulated for 24 hr using either 100 U/ml rIL-1$\alpha$, 100 U/ml rTNF$\alpha$ or medium alone was dialysed against 0·05 M Tris-HC1, pH 8·0 and applied to a $4 \times 4$ mm Heparin Sepharose column (Heparin Sepharose CL-6B; harmacia, Uppsala, Sweden). The column was washed with 10 ml 0·1 M NaCl in Tris-buffer. IL-8 activity was eluted with 10 ml 0·4 M NaCl in Tris-buffer. The eluted fraction was concentrated by ultrafiltration to 2 ml (10% of initial volume), dialysed against RPMI-1640 and filter sterilized. In repeated experiments, more than 90% of IL-8 activity in the conditioned media could be recovered. Neither IL-1 nor TNF binds to heparin Sepharose under these conditions.

In vivo *studies of the effect of cytokines*

Recombinant IL-8 (with $2 \times 10^6$ U/mg of neutrophil chemotactic activity: Furuta *et al.*, 1989), 1–100 ng/ml, diluted in PBS, was injected intradermally into the ears of Fisher rats as described elsewhere (Larsen *et al.*, 1989). Likewise, rIL-1$\alpha$ (1–1000 U/ml) or rTNF$\alpha$ (1–1000 U/ml) was injected intradermally. All recombinant cytokines were free from endotoxin and diluted into endotoxin-free PBS (< 0·05 ng/ml LPS as measured by the limulus amoebocyte lysate assay). Biopsies were taken 3 hr after injection of cytokines and tissue was prepared for histological examination as described earlier (Larsen *et al.*, 1989).

## RESULTS

The capacity of the cytokines to induce fibroblasts to express mRNA for IL-8 was evaluated using Northern blotting techniques. Figure 1a-d shows the autoradiography of total RNA from fibroblasts hybridized with a $^{32}$P-labelled IL-8 cDNA-probe used to detect the expression of mRNA for IL-8. Time-course studies (Fig. 1a, c) showed rapid induction of mRNA for IL-8 (within 30 min) when stimulated with either 100 U/ml IL-1$\alpha$ or 100 U/ml TNF$\alpha$. Note that the mRNA expression at 0 hr was undetectable. In the left lanes in Fig. 1 a–d are positive controls showing mRNA induction of IL-8 by LPS-stimulated human PBMC (Matsushima *et al.*, 1988). Dose-response experiments (Fig. 1b, d) demonstrated induction of mRNA for IL-8 using either IL-1$\alpha$ or TNF$\alpha$ concentrations as low as 0·1 U/ml when stimulating for 3 hr. We failed to observe induction of IL-8 by fibroblast growth factor or epidermal growth factor (data not shown).

We also measured the release of biological activity from fibroblasts. Table 1 compares the levels of neutrophil chemotactic activity in partially purified culture supernatants from unstimulated fibroblasts, IL-1- and TNF-stimulated fibroblasts, respectively. As a positive control we included 100 ng/ml IL-8. The results are given as chemotactic index (CI) $\pm$SD ($n=3$), i.e. the number of migrating cells in the experimental sample divided by the number of migrating cells in medium alone. As seen in Table 1, significant neutrophil chemotactic activity was released when fibroblasts were stimulated with optimal doses of rIL-1$\alpha$ or rTNF$\alpha$. TNF$\alpha$-stimulated fibroblasts produced less neutrophil chemotactic activity than IL-1-stimulated fibroblasts. No significant neutrophil chemotactic activity was produced by unstimulated fibroblasts.

Likewise we tested the induction of IL-8 mRNA in cultured keratinocytes. We observed a significant induction of IL-8 mRNA in response to 1000 U/ml IL-1$\alpha$ for 2 hr (Fig. 2). No significant induction was obtained when using up to 1000 U/ml TNF$\alpha$ for 2 hr (data not shown).

In order to evaluate the relationship of the *in vivo* inflammatory effects of IL-8, IL-1 and TNF, the cytokines were separately injected into the ears of Fisher rats. We have already reported the dose-dependent migration of neutrophils and lymphocytes into intradermal injection sites of rIL-8 in rat ears (Larsen *et al.*, 1989). Figure 3a shows the absence of polymorphonuclear leucocytes and lymphoid cells in the connective tissue surrounding post-capillary venules of sham-inoculated rat ears. Dose-dependent margination and diapedesis of lymphocytes and polymorphonuclear leucocytes in post-capillary venules was induced by intradermal injection of 10–1000 U/ml of rIL-1$\alpha$. Sparse infiltration of leucocytes was induced by 10 U rIL-1$\alpha$ but both lymphoid and polymorphonuclear cells were induced to emigrate (Fig. 3b). One-hundred U/ml rIL-1$\alpha$ induced emigration and infiltration consisting predominantly of polymorphonuclear leucocytes, although rare lymphoid cells were also present (Fig. 3d). One-thousand U/ml rIL-1$\alpha$ induced a similar degree of leucocyte infiltration except that fewer lymphoid cells were present (data not shown). Thus, polymorphonuclear cells were observed in the infiltrate at all doses, while IL-1 did not

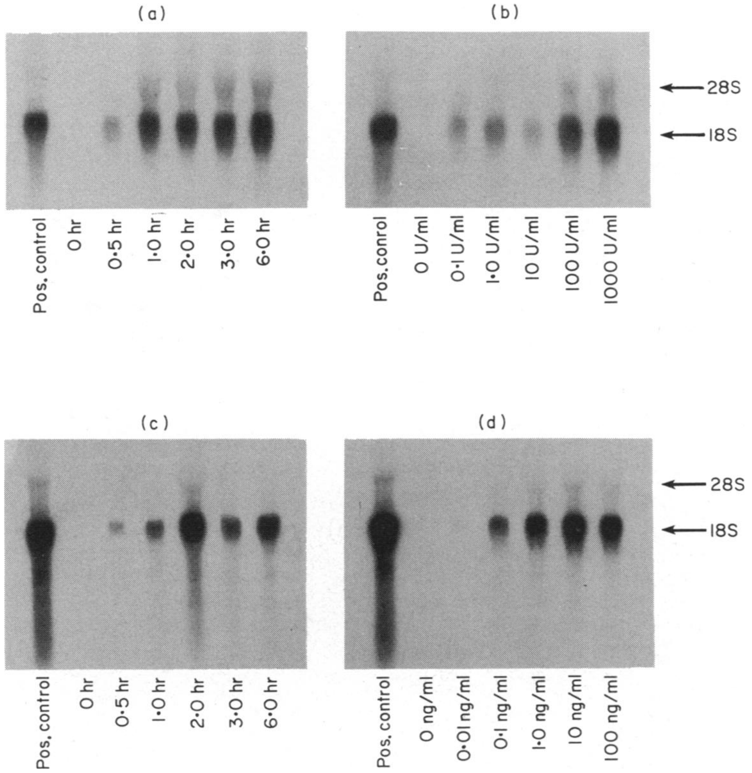


**Figure 1.** Induction of mRNA for IL-8 in normal fibroblasts, stimulated with either rIL-1α or rTNFα (1 ng = 10 U) at different doses and time intervals. The left lane of (a)–(d) shows the induction of mRNA in LPS-stimulated normal human PBMC as positive controls. The presence of equal amounts of total RNA in each lane was confirmed by a separate ethidium bromide-stained gel. The arrows indicate the positions of the 18 and 28 S fragments. (a) Shows the induction of mRNA for IL-8 in fibroblasts stimulated for 0–6 hr with 100 U/ml rIL-1α. (b) Fibroblasts stimulated for 3 hr with 0·1–1000 U/ml rIL-1α. (c) Fibroblasts stimulated for 0–6 hr with 100 U/ml rTNFα. (d) Fibroblasts for 3 hr with 0·1–1000 U/ml rTNFα.

**Table 1.** Neutrophil chemotactic activity of culture media from normal human dermal fibroblasts

| Source of chemoattractant | CI | SD | |
|---|---|---|---|
| Unstimulated fibroblasts | 0·81 | 0·29 | |
| rIL-1-stimulated fibroblasts | 6·20 | 0·55 | ($P<0·05$) |
| rTNF-stimulated fibroblasts | 3·43 | 0·58 | ($P<0·05$) |
| rIL-8 (100 ng/ml) | 8·05 | 0·70 | ($P<0·05$) |

Neutrophil chemotactic activity present in 1 : 100 dilution of partially purified culture media from normal human dermal fibroblasts stimulated with either 100 U/ml IL-1α or 100 U/ml rTNFα for 24 hr. Culture medium from unstimulated fibroblasts was also included. As a positive control we included IL-8 (100 ng/ml). Neutrophil chemotactic activity is shown as chemotactic index (CI). CI = 1·0 indicates no chemotactic activity. SD indicates standard deviation ($n = 3$) and statistical analysis was performed using a Student's *t*-test. These data shown represent three independent neutrophil chemotactic assays.

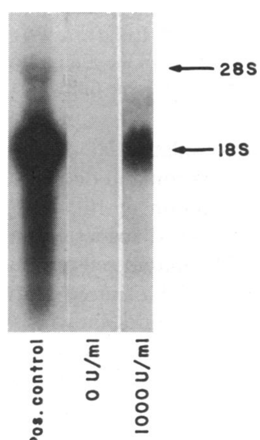


**Figure 2.** Induction of mRNA for IL-8 in normal human keratinocytes, stimulated with 0 or 1000 U/ml rIL-1α for 2 hr. First lane shows positive control, LPS-stimulated human PBMC. Arrows indicate the positions of the 18 and 28 S fragments.

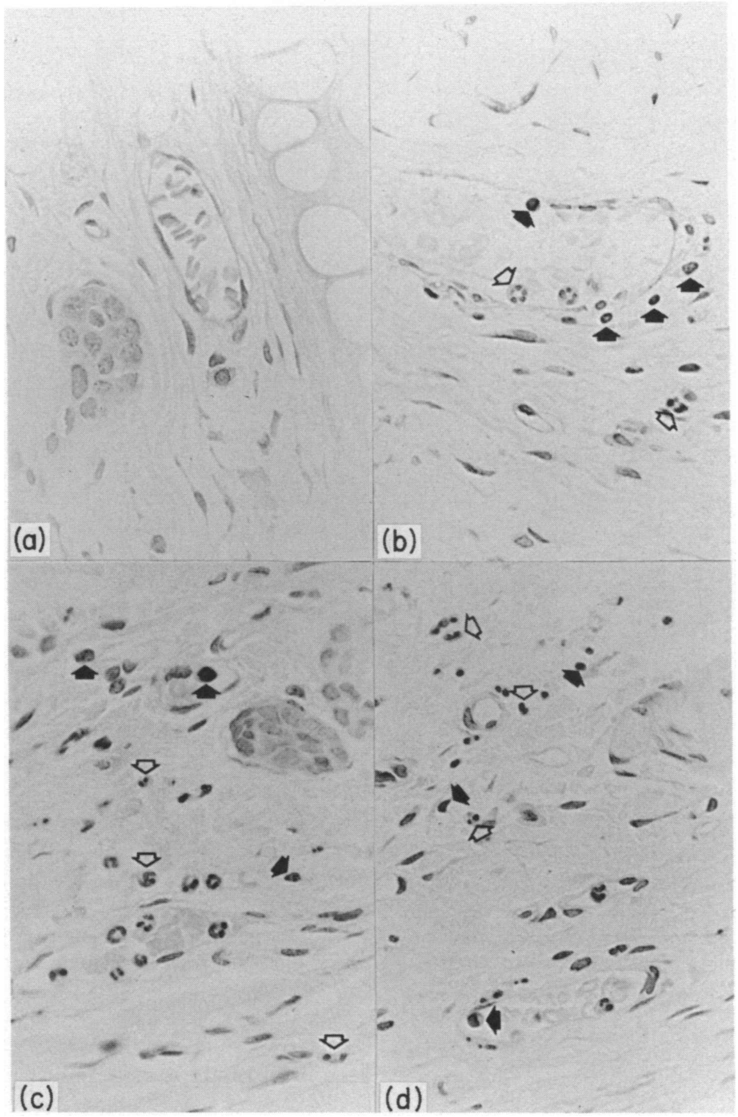


**Figure 3.** The histological appearance ( × 780) of rat ear dermis after sham injection (a) and 3 hr after intradermal injection (10 $\mu$l) of (b) 10 U/ml IL-1$\alpha$, (c) 100 U/ml rTNF$\alpha$, and (d) 100 U/ml rIL-1$\alpha$. Tissue sections were prepared routinely and stained with haematoxylin and eosin. Lymphoid cells (solid arrows) and polymorphonuclear cells (open arrows) are present in different proportions as a function of the injected dose of cytokine. Emigrating neutrophils are recognizable, but their morphology is distorted as they insinuate between pericytes and connective tissue fibres.

cause lymphocyte accumulation at highest doses. TNF$\alpha$ also caused leucocyte margination and diapedesis with an optimum effect at 100 U/ml. Injection of 10 U/ml rTNF$\alpha$ caused less leucocyte margination and diapedesis, but induced a cellular infiltrate consisting of lymphoid cells and neutrophils, whereas injection of 1000 U/ml rTNF$\alpha$ caused greater leucocyte margination than infiltration (data not shown). Whether neutrophils or lymphocytes were elicited by IL-8 was dose-dependent (Fig. 4a–d). In contrast, the inducers of IL-8, i.e. IL-1 or TNF, generally induced a mixture of both neutrophils and lymphocytes over a wide dose range.

## DISCUSSION

In the present report we show that IL-8 can be produced by non-circulating extravascular cells such as keratinocytes and dermal fibroblasts, thus further demonstrating that there are multiple cell sources for IL-8 (Yoshimura *et al.*, 1987a; Schroder *et al.*, 1988; Schroder & Christophers, 1989).

We used two inflammatory cytokines, IL-1 and TNF, previously reported to induce IL-8 in human PBMC (Matsushima *et al.*, 1988), as stimulants for IL-8 production in fibroblasts and keratinocytes, and observed that these cytokines were potent inducers of IL-8 production in fibroblasts. Keratinocytes, however, seemed less sensitive to IL-1 and, in case of TNF, we observed no significant IL-8 induction in keratinocytes. This difference in IL-1 and TNF sensitivity between the two cell types may be due to the presence of hydrocortisone in the keratinocyte growth medium, possibly modifying IL-8 production by keratinocytes and/or inhibiting the responsiveness of cells to TNF. Apart from IL-1 and TNF, several other stimulants also have been reported to stimulate the production

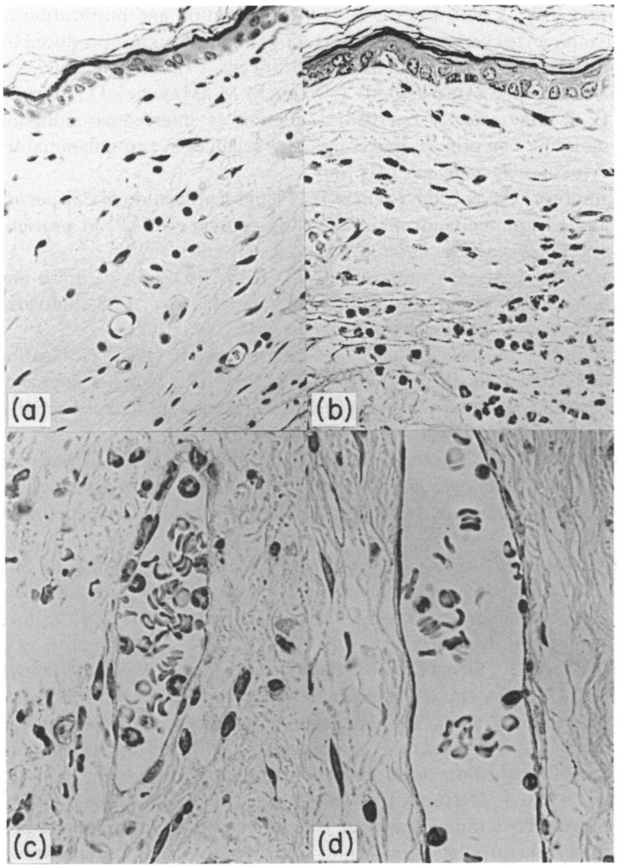


**Figure 4.** Histological sections (a) and (b) (×260) (c) and (d) (×975) of rat ear skin at sites of IL-8 injection. Low doses (0·001 μg/ml) of rIL-8 produced predominantly lymphocyte infiltrates (a) while high doses (0·1 μg/ml) of rIL-8 leads to a neutrophilic response (b). An intermediate dose of 0·01 μg/ml rIL-8 effects both cell types in a gradient-like manner. Thus, near the injection site (one high power field from the needle track) there is a predominant neutrophil margination and diapedesis (c), while lymphocyte diapedesis predominates two or more high power fields away from the injection site (d) (unpublished histological data; Larsen *et al.*, 1989).

of IL-8. Thus, Schroder *et al.* (1987, 1988) demonstrated the production of IL-8-like polypeptides from mitogen (PHA or Con A)-stimulated lymphocytes and from LPS-stimulated endothelial cells.

We have previously reported that IL-1 lacks *in vitro* neutrophil chemotactic activity (Yoshimura *et al.*, 1987a). We observed that human rTNFα enhances neutrophil migration *in vitro*, but only at high concentrations (> 1000 U/ml) (data not shown). However, a recent report (Cybulsky, Movat & Dinarello, 1987) demonstrates that intradermal injection of IL-1 as well as TNF causes a pronounced accumulation of neutrophils, at the site of injection. We have confirmed this for both human rIL-1α, and rTNFα and our data shows that IL-1α, TNFα and IL-8 have similar *in vivo* effects on the local accumulation of polymorphonuclear leucocytes. This suggests that the inflammatory effects of IL-1 and TNFα may be mediated by IL-8. However, this may not be the case because in contrast to IL-8, both rIL-1α and rTNFα caused an accumulation of a mixture of polymorphonuclear leucocytes and lymphocytes even at the lowest doses, while IL-8 at low doses resulted predominantly in a lymphocytic infiltrate. This difference between IL-1, TNFα and IL-8 could be explained by a marked induction of IL-8 production from fibroblasts and keratinocytes following injection of even small doses of IL-1 and TNF. Alternatively, the *in vivo* effect of IL-1 and TNF may be different from that of IL-8 since IL-1 and TNF also induce cell adhesion molecules, e.g. E-LAM and I-CAM (Rothlein *et al.*, 1988; Pober *et al.*, 1986), as well as inflammatory mediators, e.g. prostaglandins and leukotrienes (Elias *et al.*, 1987), which could also be involved in *in vivo* leucocyte migration. In contrast, IL-8 does not induce I-CAM or E-LAM expression on endothelial cells, but induces Mac-1 expression on human neutrophils (Farina *et al.*, 1989). Therefore, IL-1/TNF and IL-8 may interact in eliciting leucocyte migration *in vivo*.

The pathophysiological relevance of IL-8 production by non-leucocytic cells still needs to be established. Perhaps non-migrating cells such as dermal fibroblasts and keratinocytes are stimulated to produce IL-8 in response to IL-1 or TNF in the course of tissue injury or antigenic challenge, initiating a cutaneous delayed-type hypersensitivity (DTH) reaction (Gahring, Buckley & Daynes, 1985; Kupper *et al.*, 1986; Larsen *et al.*, 1988). Infiltrating lymphocytes (Schroder *et al.*, 1987) or monocytes may in turn amplify the cytokine production or even supplement the production by damaged regional keratinocytes and fibroblasts. If IL-8 contributes to the development of a DTH reaction, our observations may also lead to an understanding of the histopathology of DTH reactions in the human skin (Gawkrodger *et al.*, 1986), which reveals that both lymphocytes and neutrophils migrate to the site of antigen challenge, but with lymphocytes appearing earlier and in higher numbers than neutrophils. Schroder & Christophers (1986) have demonstrated high levels of IL-8-like activity in neutrophil-rich psoriatic scales from human patients. Recent morphological studies on the development of psoriatic lesions (Tagami, Iwatsuki & Takematsu, 1987) showed that lymphocytes are the first cell type to migrate into the lesional area, while neutrophils dominate the subsequent leucocyte infiltration. This progression could be based on a progressive rise in the local release of IL-8. The exact contribution of IL-8 in inducing cell migration by IL-1 or TNF can be more precisely evaluated when neutralizing antibodies to rat IL-8 become available.

In conclusion, the fact that IL-8 is produced by connective tissue cells in response to proinflammatory mediators such as IL-1 and TNF suggest a role of IL-8 in local inflammatory responses.

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